0013-7227/83/1132-0711$02.00/0
Endocrinology

Vol. 113, No. 2
Printed in U.S.A.

Monoiodoglucagon: Synthesis, Purification by High Pressure Liquid Chromatography, and Characteristics as a Receptor Probe*

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ABSTRACT. The synthesis of [^{125}I-Tyr^{10}]monoiodoglucagon from glucagon and carrier-free ^{125}I using 1,3,4,6-tetrachloro-3-6-diphenylglycouril (Iodogen) and its separation in pure form by reverse phase high pressure liquid chromatography (HPLC) over C_{18}-μBondapak columns using two consecutive linear gradients between solvent A [40:60 mixture of methanol and 10 mM H_3PO_4 in H_2O (pH adjusted to 3.0 with triethylamine)] and solvent B (50:50 mixture of acetonitrile and 0.1 M Tris-HCl, pH 9.0) is reported.

The newly synthesized [^{125}I]monoiodoglucagon is shown to activate adenylyl cyclase in liver membranes with an EC_{50} between 5- and 8-fold lower than that of native glucagon. Further, it binds specifically to sites on liver plasma membranes that have the characteristics of glucagon receptors in terms of guanine nucleotide sensitivity and rates of reaction.

It is suggested that [^{125}I-Tyr^{10}]monoiodoglucagon is a suitable probe for studying structural and functional properties of glucagon receptors. (*Endocrinology* **113:** 711, 1983)

THE PREPARATION of iodoglucagon was first reported in 1971 (1). Two methods of synthesis were used depending on whether large or trace amounts of iodinated glucagon were made. For large amounts, iodination was accomplished by using I_2 as the iodinating agent; for trace amounts, iodination resulted from mixing glucagon with [^{125}I]KI and chloramine-T. Preparative polyacrylamide gel electrophoresis of reaction products obtained upon mixing glucagon with I_2 yielded a major peak of iodine that comigrated with material absorbing at 280 nm, suggestive of the formation of iodoglucagon. Bioassay of this iodinated material showed it to stimulate the glucagon-sensitive adenylyl cyclase in liver plasma membranes with a potency that was indistinguishable from that of native glucagon when concentrations were calculated on the basis of absorbance at 280 nm using as a molar absorption coefficient that of native glucagon. Molarity of glucagon or glucagon-like material calculated in this manner coincided with the molarity of the iodine atoms in the same fractions. This led to the conclusions that a monoiodoglucagon had been prepared and that the biological activity of glucagon is not altered by iodination. On the basis of these findings, trace amounts of glucagon were subsequently iodinated using carrier-free ^{125}I oxidized with chloramine-T. The [^{125}I]glucagon obtained in this manner was assumed to be a monoiodinated species of glucagon, and its molarity was calculated on the basis of its iodine content, assuming that all was biologically active and that its biological activity was equal to that of the starting glucagon. The chloramine-T-iodinated glucagon was used as a receptor probe, and two sets of data were obtained. On the one hand, iodoglucagon was found to bind with a high degree of specificity to high affinity binding sites in liver parenchymal cell plasma membranes (1), and this binding was discovered to be influenced by GTP (2). This eventually led to the conclusion that hormonal stimulation of adenylyl cyclases is absolutely dependent on guanine nucleotides (3) and that adenylyl cyclases are two-component systems (4, 5). On the other hand, it was found that both association and dissociation rates of iodoglucagon to and from the membranes were too slow to account for the rates at which adenylyl cyclase activity was affected (6). The thought was expressed that slow binding reaction rates might be indicative of the existence of a large proportion of the total glucagon-specific sites being permanently uncoupled from the adenylyl cyclase system (6). This interpretation assumed that a chemically homogeneous receptor probe had been used.

Work by Bromer *et al.* (7) subsequently showed that

Received November 29, 1982.
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* This work was supported in part by NIH Grants AM-19318 and NS-15950.
† Supported by NIH Diabetes Training Grant AM-27685. Deceased November 17, 1982.

iodinated species of glucagon are between 4–10 times more potent in adenylyl cyclase assays than native glucagon, the potency depending on the degree of iodination. This was confirmed in independent work by Desbuquois (8). This indicated that the original report that iodinated glucagon isolated by preparative polyacrylamide gel electrophoresis is equipotent with native glucagon was erroneous and suggested that such preparations of iodinated glucagon may be composed of a mixture of glucagon derivatives of which only a small fraction are biologically active iodoglucagon molecules, with the remainder being one or more species of glucagon that do not activate adenylyl cyclase, reducing the potency of the total mixture, but that bind to liver membranes with anomolous kinetics, creating the impression of the existence of heterogenous sets of sites. It is also clear from the above data that the resolution capacity of the preparative polyacrylamide gel electrophoresis method used was insufficient to separate an active monoiodoglucagon species usable as probe in receptor studies.

In the present work, we have applied high pressure liquid chromatography (HPLC) on C_{18} reverse phase columns to separate labeled products that result upon exposure of glucagon to iodination procedures and have developed a new iodination procedure which allowed us to separate one peak of iodinated glucagon, of which we described the properties in terms of number of iodines per molecule, localization of iodine, biological potency, and receptor affinity. It is shown that monoiodoglucagon interacts with a single class of binding sites on liver plasma membranes and that the reaction kinetics are such to fully warrant the assumption that all of the sites with which [^{125}I]monoiodoglucagon interacts are receptors that activate adenylyl cyclase.

Materials and Methods

Materials

Carrier-free ^{125}I was purchased from Isotex Diagnostics (Friendswood, TX). Inorganic carrier-free ^{32}P used for synthesis of [α-^{32}P]ATP was purchased from Union Carbide (Tuxedo, NY). Glucagon (recrystallized; insulin-free) was a generous gift from Dr. W. W. Bromer, Eli Lily and Co. (Indianapolis, IN). Stock suspensions (10 mg/ml) of this material were made in 10 mM cetyltrimethylammonium chloride (Eastman Kodak Co., Rochester, NY) and diluted with distilled water to the desired concentration before use. Iodogen (1,3,4,6-tetrachloro-3-6-diphenylglycouril) was purchased from Pierce Chemical Co. (Rockford, IL) and used after plating it onto the bottom of a 10 × 75-mm glass test tube. This was accomplished by adding appropriate volumes of a 4.0 mg/ml solution of Iodogen in chloroform to the bottom of the tubes to be plated and evaporating the solvent under a gentle stream of nitrogen. For the experiments described in this report, glass-plated Iodogen was prepared fresh before use. Oxoid cellulose-acetate filters were obtained from Oxoid Ltd. (Basingstoke, Hants, England) and soaked for 2–10 h in 10% BSA before use. [α-^{32}P]ATP was synthesized by the method of Walseth and Johnson (9) and purified by ion exchange chromatography over DEAE-Sephadex A-25, as described previously (10). GTP was obtained from Sigma Chemical Co. (St. Louis, MO). C_{18} reverse phase columns (μBondapak C_{18}; 0.4 × 3 cm) were purchased from Waters Associates (Waltham, MA). Methanol and acetonitrile used for HPLC were obtained from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). BSA (Cohn fraction V) was purchased from Armour Pharmaceutical Co. (Phoenix, AZ). All BSA-containing solutions were filtered through 0.45-μm Millipore HA type filters (Millipore Corp., Bedford, MA) before use. All other materials and chemicals were of the highest purity commercially available. Their sources were recently described (11).

Iodination of glucagon

Unless described otherwise, 9–11 nmol glucagon in 90 μl dilution medium (described under *Materials*) were iodinated by mixing with 90 μl 0.4 M potassium phosphate buffer, pH 7.2, and 60 μl 20 mCi carrier-free ^{125}I in 0.1 N sodium hydroxide (~9 nmol), followed by transfer of this mixture into a 10 × 75-mm glass test tube with 4.0 μg (9 nmol) Iodogen plated onto its bottom. The reaction was stopped after 1.0 min at room temperature by the addition of 10 μl 2 mM tyrosine and 10 μl 2 mM freshly made sodium metabisulfide, followed by cooling to 0–4 C.

Reverse phase HPLC

Reverse phase HPLC was performed on Waters μBondapak-C_{18} columns (0.4 × 30 cm) was performed at a constant flow rate of 1.0 ml/min using the following solvents: a 40:60 mixture of methanol and 10 mM H_3PO_4 in H_2O, adjusted to pH 3.0 after mixing with freshly redistilled triethylamine (solvent A), and a 50:50 mixture of acetonitrile and 0.1 M NH_4HCO_3 in H_2O or 0.1 M Tris-HCl, pH 9.0, in H_2O (solvent B). The column received the solvents from one or two Waters model 6000A pumps directed by Waters model 660 gradient programmer. Injections were made without interruption of pressure or flow using a Waters model U6K injector located between the solvent pumps and the column. Column eluates were monitored for absorption at 254 nm with the aid of a Waters model 440 absorption detector fitted with an 8 μl flow cell and then fractionated with the aid of a Gilson fraction collector (Gilson Medical Electronics, Middleton, WI). Chromatography, UV monitoring, and fraction collection were carried out at room temperature (22–25 C). Before use, the column was washed with methanol (30 min), water (30 min), and solvent A (30 min). Next, samples to be chromatographed (dissolved in methanol, water, or solvent A) were injected onto the head of the column and, after washing with solvent A for 5–30 min, the chromatographic separation was effected by subjecting the column to a nonlinear gradient between solvents A and B, formed of two consecutive linear gradients of which the second was 50% (acetonitrile-0.1 M NH_4HCO_3 as solvent B) or 12.5% (acetonitrile-0.1 M Tris-HCl as solvent B) as steep as the first For further details see figure legends. As illustrated in Fig. 1,

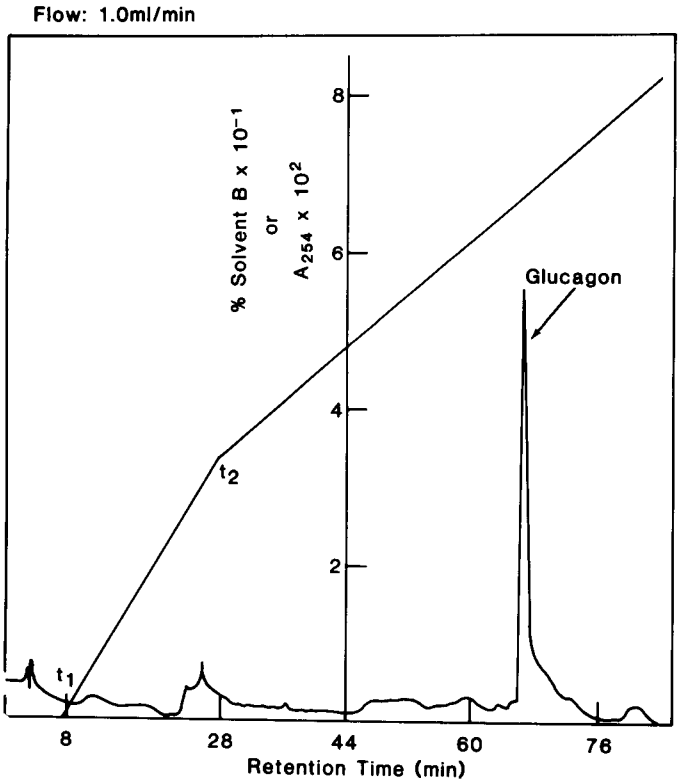

FIG. 1. Reverse phase HPLC of native glucagon. Ten nanomoles of glucagon in 25 μl water were injected onto the head of the μBondapak-C_{18} column previously equilibrated at 1.0 ml/min with solvent A. Back pressure was about 1200 psi. After washing for 12 min, the column was subjected to two consecutive linear gradients between solvents A and B. The first one was a linear gradient from 0–33% solvent B from t_1 = 8 min to t_2 = 28 min, and the second one was a linear gradient from 33–100% from t_2 = 28 min to t_3 = 108 min. Column eluates were monitored for adsorption at 254 nm. Absorbance units full scale (AUFS) = 0.1 from 0–32 min, and AUFS = 0.01 from 32–90 min. Glucagon eluted from the column essentially as a single sharp peak at about 68% solvent B and approximately 70 min after application to the column.

under these conditions, native glucagon elutes as a single sharp peak. This position varied somewhat with the age of the column and previous uses for other purposes, but was very reproducible among five to eight consecutive runs. To minimize adsorption artifacts at 254 nm throughout the elution procedure, the column was subjected to a prerun, *i.e.* a blank gradient elution followed by a 30-min equilibration period with solvent A before injecting the sample to be chromatographed. After gradient elution, the column was washed a minimum of 30 min with solvent A, then used again or washed further with water (60 min) and methanol (60 min), in which it was finally stored.

If fractions were rechromatographed, they were first diluted 4-fold with water and then reinjected onto the head of the lumn equilibrated in solvent A. The injection volumes of samples to be rechromatographed varied and were as large as 1.6 ml. If the material needing rechromatography was more than 1.6 ml, it was injected via repetitive cycles of 1.6 ml, followed by 8 ml (8 min) solvent A, until all of the sample had been applied. Chromatographic separation was then effected by the nonlinear gradient elution procedure described above.

Storage of iodinated glucagon purified by HPLC and preparation for use in binding assays

HPLC-purified iodinated glucagon was aliquoted into 150- to 200-μl fractions and stored at −70 C until used. On the day of use, the solvent (32% solvent A-68% solvent B) was changed to 0.1% BSA, 1.0 mM EDTA, and 20 mM Tris-HCl, pH 7.5 (buffer I), by passing up to 450-μl aliquots over a Sephadex G-10 (fine) column of 7-ml bed volume made in a Mohr measuring 5-ml glass pipette equilibrated in buffer I. The excluded radioactive peak was collected and filtered through 0.45-μm Millipore HA filters of 13 mm diameter supported by a Millipore Swinny adaptor (catalog no. XX3001200). Depending on the age of the iodinated glucagon preparation, up to 40% of the iodine was lost on the filter. After filtration, the molarity of the iodinated glucagon solution was calculated on the basis of ^{125}I content, assuming the material to be composed of monoiodoglucagon (for justification, see *Results*).

Binding of iodinated glucagon to liver plasma membranes and separation of free from bound hormone

Unless stated otherwise, liver membranes prepared by the abbreviated (12) method of Neville, Jr. (13), were incubated at a final concentration of 0.02–0.25 mg protein/ml in a final volume of 100 μl buffer I and the indicated amounts of freshly filtered ^{125}I-labeled glucagon, guanine nucleotides, Mg ion, and other additions. After incubation at the indicated temperatures and times, the reactions were stopped following essentially the method originally described by Goldfine *et al.* (14). Briefly, 5 ml ice-cold buffer I were added to the test tubes, and the mixtures were vortexed and immediately filtered through 0.45-μm Oxoid filters that had been presoaked in 10% BSA and washed with ice-cold buffer I immediately before receiving the stopped and diluted reaction mixture. The tubes were rinsed once with 5 ml ice-cold buffer I, and the rinses were filtered through the Oxoid filters, which were then immediately washed with a final 5-ml aliquot of ice-cold buffer I. The whole procedure took no more than 20 sec. The amount of radioactivity remaining on the Oxoid filters was quantitated by scintillation counting in a Searle Analytic (Des Plaines, IL) model 1195 autogammaspectrometer. Under the above conditions and using 0.5 nM [^{125}I]monoiodoglucagon prepared as described above, the nonspecific binding measured in the presence of 10–15 μg membrane protein and an excess of unlabeled glucagon was about 2% of the total counts added and about 10% of the total counts bound to membranes. In general, the nonspecific binding value was nearly identical to the value obtained when binding to Oxoid filters was measured in the absence of membranes.

Adenylyl cyclase assay and determination of potencies of glucagon and iodinated glucagons

Glucagon or iodinated glucagon obtained by HPLC fractionation (in solvent A-solvent B mixture) were diluted serially in

ice-cold buffer I to yield final concentrations between 10^{-11} and 10^{-7} M. The molarity of the initial solution of glucagon dissolved in distilled water was calculated from its absorbance at 280 nm using a molar absorption coefficient of 8050. Molarity of HPLC-purified radioactive glucagon was assumed to equal that of ^{125}I (for justification, see *Results*). Aliquots of the serially diluted sample (10 μl) were then tested for their capacity to stimulate adenylyl cyclase activity in 2–6 μg rat liver plasma membranes, as assayed in 10 min at 32.5 C in a final volume of 50 μl containing (final concentrations) 0.1 mM [α-^{32}P]ATP (~2000 cpm/pmol); 5 mM $MgCl_2$, 10 μM GTP; 1.0 mM EDTA; 0.1% BSA; 1.0 mM [^{3}H]cAMP (~10,000 cpm); a nucleoside triphosphate-regenerating system composed of 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase (200 U/mg), and 0.02 mg/ml myokinase (200 U/mg); and 25 mM Tris-HCl, pH 7.5. Some reactions were carried out in the presence of 0.5 mM AMP-P(NH)P and 2×10^7 cpm [α-^{32}P]ATP (~1000 cpm/pmol) instead of 0.1 mM [α-^{32}P]ATP and the nucleoside triphosphate-regenerating system described above, with the remainder of the reaction mixture kept the same. The reactions were stopped, and cAMP formed from ATP was determined by the method of Salomon *et al.* (15), as modified by Bockaert *et al.* (16).

Trypsin digestion of HPLC-purified iodinated glucagon and chromatography of reaction products

Iodinated glucagon was passed over Sephadex G-10 as described above. Aliquots containing between 10^6 and 10^7 cpm ^{125}I were then incubated overnight at 32.5 C in a final volume of 0.2 ml with 40 μg/ml crystalline trypsin (catalog no. 3703, Worthington Biochemical Corp., Freehold, NJ) in medium containing 1 mM $CaCl_2$, 0.1% BSA, and 25 mM Tris-HCl, pH 7.6. At the end of these incubations, the resulting mixtures were applied onto a 13 × 1.1-cm column of Sephadex G-25 (superfine) made in a 5-ml Mohr measuring glass pipette equilibrated with 0.5 M acetic acid. The column was developed with the same solvent, six-drop aliquots of approximately 300 μl each were collected, and the size distribution of the ^{125}I-containing fragments was determined by scintillation counting, as described by Bromer *et al.* (17).

Pronase digestion of HPLC-purified iodinated glucagon and chromatography of reaction product

Iodinated glucagon was passed over Sephadex G-10, as described above. Aliquots containing 10^6–10^7 cpm ^{125}I were then incubated with 10 mg/ml pronase (catalog no. 53702, Calbiochem-Bering Corp., San Diego, CA) for 36 h at 32.5 C in medium containing Tris-HCl, pH 7.5. At the end of the incubation, tyrosine, monoiodotyrosine, diiodotyrosine, and monoiodohistidine were added as marker amino acids. The proportion of total radioactivity behaving as monoiodohistidine, monoiodotyrosine, or diiodotyrosine was then determined by ion exchange chromatography, as described by Savoie *et al.* (18). Briefly, the resulting mixture was applied onto a 13 × 1.1-cm Dowex AG1-X4, chloride form, 200- to 400-mesh column equilibrated with water. The column was then developed with a mixture of 0.1 N HCl-butanol (3.3%, vol/vol), and fractions (0.25 ml each) were collected. Elution profiles of radioactivity were determined by scintillation counting and compared to those of marker monoiodohistidine, monoiodotyrosine, and diiodotyrosine determined by measuring UV absorbance.

All other procedures, methods, and materials used throughout have been described recently (11).

Results and Discussion

Iodination and separation of iodinated products

Based on our experience with reverse phase HPLC separating iodinated hydroxybenzylpindolols from noniodinated hydroxybenzylpindolol (19), and encouraged by reports from the laboratory of J. Revier (20) and an article by Hancock *et al.* (21) indicating the appropriateness of using this separation technique for peptide hormones, we set out to develop a technique that might be useful in separating iodoglucagon(s) from native glucagon. The chromatographic scheme illustrated in Fig. 1, showing native glucagon eluting at 66–68% of solvent B, appeared acceptable to test for separation of iodination products.

When iodoglucagon preparations, obtained as described earlier (1, 6) by iodinating with chloramine-T as the oxidizing agent and purifying by absorption to and desorption from cellulose (1), were subjected to reverse phase HPLC, we obtained elution profiles of radioactivity that are best described as smears, *i.e.* formed of a very broad set of many peaks, most of which eluted between the initiation of the second linear gradient and the elution of native glucagon, with very few counts per min eluting later than the starting native material (not shown). Replacement of chloramine-T by the combination of lactoperoxidase and H_2O_2 did not improve matters. Further, when tested for their capacity to bind specifically to liver membranes, only peaks eluting close to native glucagon were able to do so. The smears were not artifacts of the separation technique, since upon rechromatography of aliquots from these fractionations, the radioactivity eluted in discrete peaks and in the same position of the gradient from which they had been obtained (not shown). These experiments indicated that the glucagon molecule is extremely sensitive to oxidative breakdown and that under the standard iodination conditions, such as used by several laboratories to prepare an ^{125}I probe to explore glucagon receptor function, much of the material obtained is severely damaged and unable to bind specifically to receptors, with the rest being a heterogeneous mixture of iodinated forms of undefined composition. By reducing the time of iodination to 15 sec, changing the glucagon: ^{125}I:chloramine-T ratio to 1:0.3:0.3, and eliminating the prepurification by cellulose adsorption, it was possible to obtain both a significant reduction and even a disappearance of ^{125}I-labeled ma-

terials that would not bind to liver membranes, with a concomitant appearance of three peaks of radioactive material eluting after native glucagon. However, such results were variable from time to time, and the yields of any one of the radioactive peaks was not more than 1%.

Better and more reproducible results were obtained using as an iodide-oxidizing agent the water-insoluble chloramide Iodogen. Figure 2 illustrates the elution pattern of radioactivity obtained upon reverse phase HPLC of the reaction products formed after iodinating glucagon for 1.0 min at molar ratios of glucagon to ^{125}I to Iodogen of 1:1:1 (see *Materials and Methods*). Routinely, six or seven discrete peaks were obtained. The first peak represents free unincorporated ^{125}I and constitutes between 20–25% of the total. Other peaks eluted before native glucagon, one peak eluted where native glucagon would have appeared, and two peaks eluted after native glucagon. Although the relative abundancy of any one of these peaks was found to vary by as much as ±50% from what is shown in the example of Fig. 2, their appearance and positions of elution with respect to the gradient were constant from iodination to iodination. All of the major peaks shown in Fig. 2 to elute before the elution position of native glucagon bound to liver membranes with about equal affinity and activated adenylyl cyclase with about equal potency (not shown). In contrast, the material present in what is labeled peak A was found to bind to liver membranes with higher affinity and to activate adenylyl cyclase with higher potency than native glucagon (see below). This peak represents approximately 5% of the total radioactivity used in the iodination reaction and applied to the column. It was further used without rechromatography for the experiments reported below.

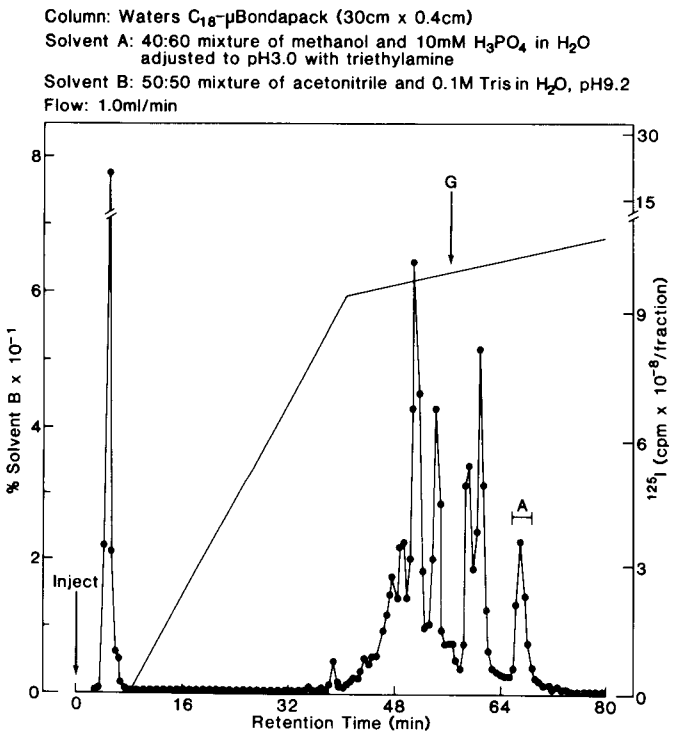

FIG. 2. Reverse phase HPLC of glucagon iodination products using Iodogen as the oxidizing agent. Elution pattern of ^{125}I-labeled materials obtained after iodinating glucagon at molar glucagon to ^{125}I to Iodogen ratios of 1:1:1 using 20 mCi ^{125}I. The reaction was allowed to proceed for 1 min at room temperature, after which the reaction was stopped, and the soluble mixture was injected onto the μBondapak-C_{18} column and subjected to the HPLC procedure, as described in the text. Fractions (450 μl) were collected, and the radioactivity of each fraction was quantitated. The *arrow* indicates the elution position of native glucagon. The last peak eluting to the right of native glucagon is shown as peak A.

Composition of peak A iodinated glucagon

Iodine in peak A is trichloroacetic acid precipitable, and on the basis that this occurred upon oxidation with Iodogen in the presence of glucagon, we conclude that radioactivity in peak A represents ^{125}I incorporated into glucagon and that peak A contains, therefore, one or more species of iodinated glucagon. We explored whether ^{125}I was incorporated into tyrosine or histidine. Extensive digestion of a sample of peak A with pronase, followed by ion exchange chromatography, showed that more than 95% of the ^{125}I comigrated with monoiodotyrosine (Fig. 3). Glucagon contains two tyrosine residues: one in position 10 and another in position 13. We explored whether the radioactivity in peak A was associated with one, the other, or both of the tyrosines of glucagon. Bromer's laboratory (17) has shown that digestion of glucagon with trypsin yields three peptide fragments: TS-1, TS-2, and TI. TS-1 and TS-2 are acid-soluble peptides corresponding to glucagon$_{1-12}$ and glucagon $_{13-17}$. TI is an acid-insoluble residue of glucagon$_{18-29}$. Gel filtration over Sephadex G-25 leads to separation of intact glucagon, TS-1, and TS-2 (17) and, hence, provides an assay for the distribution of radioactivity between the two types of tyrosine residues. As shown in Fig. 4, peak A contains iodinated glucagons with ^{125}I on the tyrosine of TS-I, *i.e.* on tyrosine 10. We conclude from these findings that peak A is composed mainly of [^{125}I-Tyr^{10}]monoiodoglucagon.

Biological activity of monoiodoglucagon

The potency of peak A [^{125}I-Tyr^{10}]monoiodoglucagon in stimulating liver plasma membrane adenylyl cyclase (molarity calculated on the basis of ^{125}I content) was found in various experiments to be between 5- and 8-fold higher than that of native glucagon (molarity calculated on the basis of absorption of the stock solution at 280 nm). This finding is illustrated in Fig. 5 and agrees with the data reported by Bromer's laboratory (7) showing a similar increase in potency for iodoglucagon prep-

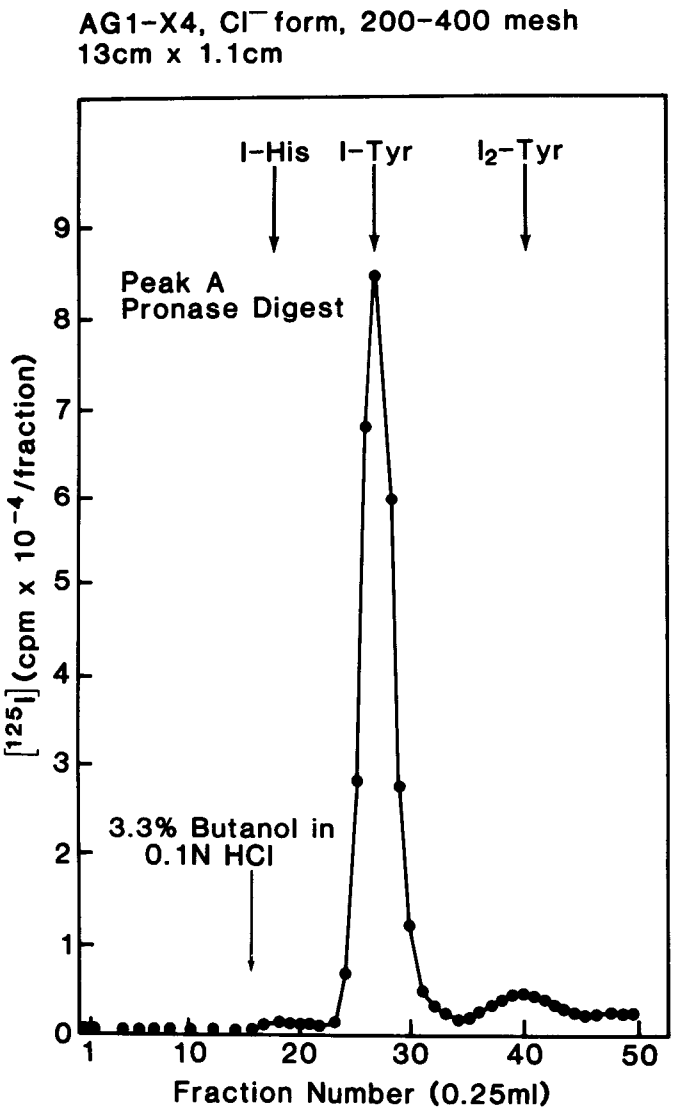

FIG. 3. Separation of pronase digest of peak A iodinated glucagon. An aliquot of the HPLC-purified peak A was digested with pronase, as described in *Materials and Methods.* The hydrolysates were then applied to a column (13 × 1.1 cm) of Dowex AG1-X4 (200–400 mesh; chloride form) and eluted with 0.1 N HCl containing 3.3% (vol/vol) butanol. The radioactive peaks were identified by reference to standard iodohistidine and iodotyrosines indicated by the arrows.

arations synthesized with nonradioactive I_2. We conclude that we have synthesized and purified ^{125}I-labeled monoiodoglucagon that is biologically active and 5- to 8-fold more potent than native glucagon. During the remainder of this report we shall refer to the radioactive, biologically active [^{125}I-Tyr^{10}]monoiodoglucagon simply as [^{125}I]monoiodoglucagon.

Binding property of [^{125}I]monoiodoglucagon

Binding of [^{125}I]monoiodoglucagon was unaffected by the addition of up to 10^{-6} M ACTH, insulin, PRL, and oxytocin (not shown). The addition of native glucagon

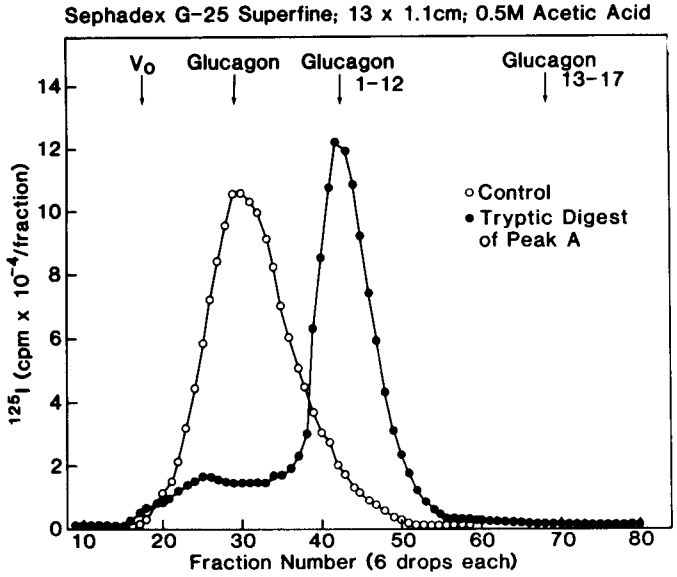

FIG. 4. Separation of peptides resulting from tryptic hydrolysis of iodinated glucagon peak A. An aliquot of the untreated HPLC-purified peak A (○) and the hydrolysates obtained after trypsin digestion of HPLC-purified peak A (●) were applied to a column (13 × 1.1 cm) of Sephadex G-25 (superfine), as described in *Materials and Methods.* Peptides were eluted with 0.5 M acetic acid, and fractions of approximately 300 μl were collected. The elution positions of intact glucagon and the two soluble peptides corresponding to glucagon$_{1-12}$ (TS-1 peptide) and glucagon$_{13-17}$ (TS-2 peptide) are shown by *arrows.* The latter was prepared by trypsin digestion of the peak immediately preceding peak A in Fig. 2. V_0, Void volume.

to a binding assay with 100,000 cpm [^{125}I]monoiodoglucagon (2400 cpm/fmol; 100 μl) competitively inhibited retention of radioactivity by the membranes giving 50% inhibition at 2.5 nM (not shown). These data indicated that the sites to which [^{125}I]monoiodoglucagon binds are specific for glucagon.

Binding of [^{125}I]monoiodoglucagon proceeds rather rapidly at temperatures above 30 C, reaching equilibrium within 1 min after the addition of 0.5–0.6 nM [^{125}I] monoiodoglucagon. The reaction rates can be reduced somewhat by reducing the temperature of incubation and are affected by guanine nucleotides. Figure 6 illustrates association rates of varying concentrations of [^{125}I]monoiodoglucagon to liver plasma membranes as obtained at 22 C in the presence and absence of 100 μM guanyl-5′-yl-imidodiphosphate [GMP-P(NH)P]. It can be seen that equilibrium binding was reached considerably faster in the presence of guanine nucleotide than in its absence (not the different time scales on *left* and *right panels* of Fig. 6) and that binding at equilibrium was less in the presence than in the absence of GMP-P(NH)P (note the different scales of y-axes on *left* and *right panels* of Fig. 6). Under both conditions, binding was unchanged for up to 30 min (not shown). On the basis of these results subsequent incubations testing for equilibrium binding

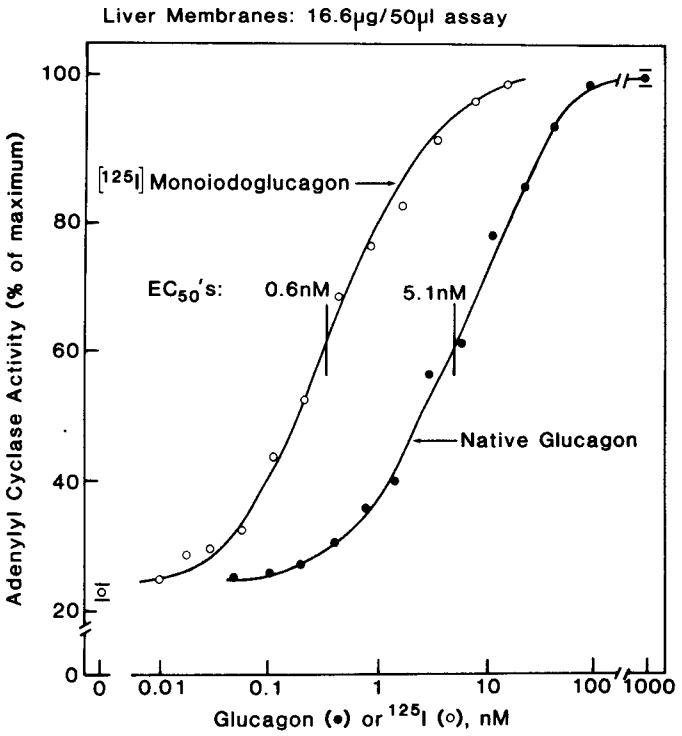

FIG. 5. Activation of liver membrane adenylyl cyclase by native glucagon and HPLC-purified [^{125}I]monoiodoglucagon. Assays were carried out in the presence of 0.1 mM [α-^{32}P]ATP (2,000 cpm/pmol), 5 mM $MgCl_2$, 10 μM GTP, 1 mM EDTA, 0.1% BSA, 1 mM [^{3}H]cAMP (10,000 m), 25 mM Tris-HCl (pH 7.5), and a nucleoside triphosphate-regenerating system consisting of 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 0.02 mg/ml myokinase. The membrane protein concentration was 16.6 μg/50 μl assay. Incubations were carried out for 10 min at 32.5 C. The concentration of iodinated glucagon was calculated on the basis of ^{125}I content, considering the material to be composed of monoiodoglucagon. The concentration of native glucagon was determined by its absorbance at 280 nm using a molar adsorption coefficient of 8050. The EC_{50}, the concentration required to obtain 50% of maximal enzymatic activity is shown by *vertical lines*.

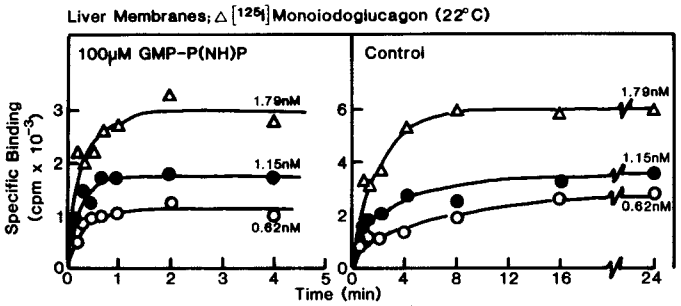

FIG. 6. Time course of specific binding [^{125}I]monoiodoglucagon to rat liver plasma membranes. Rat liver membranes (3.6 μg protein) were incubated in triplicate in a final volume of 100 μl 20 mM Tris-HCl, pH 7.5, containing 1.0 mM EDTA and 0.1% BSA (buffer I) in the presence of the indicated concentrations of [^{125}I]monoiodoglucagon (2400 cpm/fmol). Incubations were carried out at 22 C for the indicated time periods in the presence of 100 μM GMP-P(NH)P (*left panel*) as well as in the absence of added guanine nucleotide (*right panel*). Parallel assays were carried out in duplicate in the presence of 1 μM unlabeled glucagon. Assays were stopped by dilution with ice-cold buffer I and immediately filtered through Oxoid filters, as described in *Materials and Methods*. The radioactivity retained on the filters was quantitated by γ-spectroscopy. Specific binding was calculated by subtracting nonspecific binding from total binding.

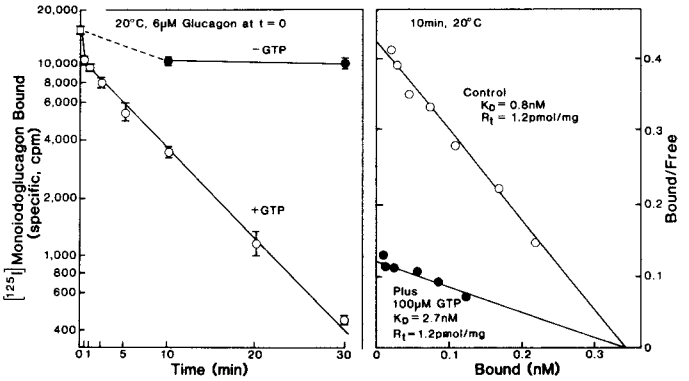

FIG. 7. Left panel, Effect of GTP on the time course of dissociation of [^{125}I]monoiodoglucagon from liver plasma membranes. Plasma membranes (25 μg protein) were incubated at 20 C for 15 min in the presence of 1 nM [^{125}I]monoiodoglucagon without added nucleotide, in a total volume of 2.0 ml. The equilibrated incubations were placed at 0–4 C for 5–10 min until the dissociation assay was initiated by the addition of 50-μl aliquots of the equilibrated samples to 5 ml dissociation medium. The dissociation medium, which was equilibrated at 20 C, consisted of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% BSA, and 6 μM unlabeled glucagon, with or without 100 μM GTP. Assays were stopped by filtration, as described in *Materials and Methods*. *Data points* represent the mean ± SD of triplicate determinations and have been corrected for nonspecific binding. *Right panel*, Scatchard analysis of equilibrium binding assays in the absence and presence of added GTP. Membranes (3.6 μg protein) were incubated with varying concentrations of [^{125}I]monoiodoglucagon in the absence or presence of 100 μM GTP. Other incubation conditions are described in Fig. 6. Specific binding data were analyzed and plotted according to the method of Scatchard.

were carried out for 10 min. Not only rates of binding but also rates of dissociation of [^{125}I]monoiodoglucagon from liver membranes are sensitive to guanine nucleotides. This is illustrated in the *left panel* of Fig. 7, where [^{125}I]monoiodoglucagon was allowed to bind to liver membranes under controlled conditions (no nucleotide added), and its rate of dissociation was subsequently assessed in a second incubation carried out after dilution and addition of excess unlabeled glucagon in the absence and presence of 100 μM GTP. Interestingly, in this and several repeat experiments, we found the dissociation reaction of [^{125}I]monoiodoglucagon to be biphasic, with about 20–25% dissociating rapidly regardless of the presence of GTP.

Equilibrium binding experiments showed that liver membranes bind [^{125}I]monoiodoglucagon in a saturable as well as in a guanine nucleotide-sensitive manner. This is illustrated in the *right panel* of Fig. 7, which presents Scatchard plots of a 20-min binding experiment at 20 C in which liver membranes were incubated with increasing concentrations of [^{125}I]monoiodoglucagon (2400 cpm/fmol). Specific binding was measured by determining the

difference between radioactivity bound when [^{125}I]monoiodoglucagon was added alone and that bound when 10^{-6} M glucagon was added to determine nonspecific binding. It can be seen that essentially linear Scatchard plots are obtained with K_d values of 0.8 nM in the absence of GTP and 2.5 nM in the presence of 100 μM GTP. Estimation of x-axis intercepts showed them to be unaffected by GTP at 1.2 pmol/mg membrane protein. Scatchard plots in the absence of GTP were also linear at temperatures up to 32.5 C (not shown). However, while the K_d value for [^{125}I]monoiodoglucagon was essentially unaltered in the absence of GTP (*e.g.* 0.7 nM at 35 C), it increased in the presence of GTP such that not enough points could be obtained to reliably define a straight line. A reliable determination of the effect of GTP on the receptor-hormone interaction at temperatures of 30 C and above will require a different approach, involving a receptor probe that is insensitive to guanine nucleotides.

Rate of activation of liver membrane adenylyl cyclase by [^{125}I]monoiodoglucagon at 22 C

As illustrated in Fig. 8, the rates of activation of liver membrane adenylyl cyclase are affected by both the concentration of hormone added and the presence of guanine nucleotide. In the absence of guanine nucleotide, the times required to obtain steady state stimulation of adenylyl cyclase with 0.22 and 0.79 nM [^{125}I]monoiodoglucagon were approximately 15 and 9 min (calculated $t_{1/2}$ values were 4.8 and 3.0 min, respectively). In the presence of 100 μM GTP and using 0.22 nM [^{125}I]monoiodoglucagon, the steady state activity was obtained much faster (calculated $t_{1/2}$, 0.5 min). These time courses of stimulation of adenylyl cyclase by [^{125}I]monoiodoglucagon (Fig. 6) correlate well with the time courses of [^{125}I]monoiodoglucagon binding to the membranes (Fig. 6). We conclude from these studies that in contrast to the previous findings with ill defined iodoglucagon (1, 2, 6), the kinetics of binding of [^{125}I]monoiodoglucagon and those of adenylyl cyclase stimulation correlate in such a manner as to justify a cause-effect relationship between the binding site occupancy and adenylyl cyclase stimulation.

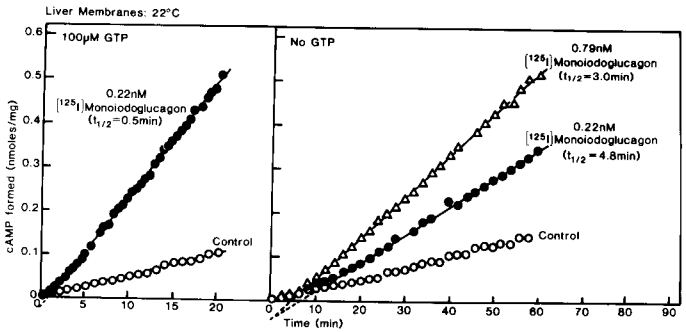

FIG. 8. Time courses of activation of liver membrane adenylyl cyclase by [^{125}I]monoiodoglucagon in the presence (*left panel*) and absence (*right panel*) of guanine nucleotide. Reactions were carried out under standard adenylyl cyclase assay conditions at the indicated concentrations of GMP-P(NH)P and [^{125}I]monoiodoglucagon. Each curve was derived from a single incubation from which 50-μl aliquots containing the cAMP formed by 3.6 μg liver membrane protein were withdrawn at the indicated times and quantitated as described in *Materials and Methods*. The $t_{1/2}$ values were calculated from the apparent activation rate constant (k) according to $t_{1/2} = \ln 2/k$; k values were calculated from the data shown according to $k = v_{steady\ state}/y$ intercept.

We conclude from the above data that the methodology presented here allows the synthesis and purification of [^{125}I-Tyr^{10}]monoiodoglucagon. Its availability should be of use in several studies, including those on variations in receptors levels and properties in disease states and elucidation of the mechanism by which the glucagon receptor activates the system(s) it couples to.

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